Attachments #12

BA

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)


(51) International Patent Classification 5 : C12N 9/64, 15/57, A61K 37/547 C12Q 1/38

A1

(11) International Publication Number: WO 91/12320

(43) International Publication Date: 22 August 1991 (22.08.91)

(21) International Application Number: PCT/US91/00912

(22) International Filing Date: 8 February 1991 (08.02.91)

(30) Priority data:
478,084 9 February 1990 (09.02.90) US



(71) Applicants: ZYMOGENETICS, INC. [US/US]; 4225 Roosevelt Way N.E., Seattle, WA 98105 (US). TEIJIN LIMITED [JP/JP]; Minamibommachi 1-chome, Chuo-ku, Osaka-shi, Osaka 541 (JP).

(72) Inventors: MIYAGI, Fuminori ; 5-18, Tamadaira 3-chome, Hino-shi, Tokyo 191 (JP). SUMI, Yoshihiko ; 40-49, Toyoda 4-chome, Hino-shi, Tokyo 191 (JP). WAKABAYASHI, Kenji ; 5-18, Tamadaira 3-chome, Hino-shi, Tokyo 191 (JP). FOSTER, Donald, C. ; 3002 N.E. 181st Street, Seattle, WA 98155 (US).

(74) Agents: MAKI, David, J. et al.; Seed and Berry, 6300 Columbia Center, Seattle, WA 98104-7092 (US).



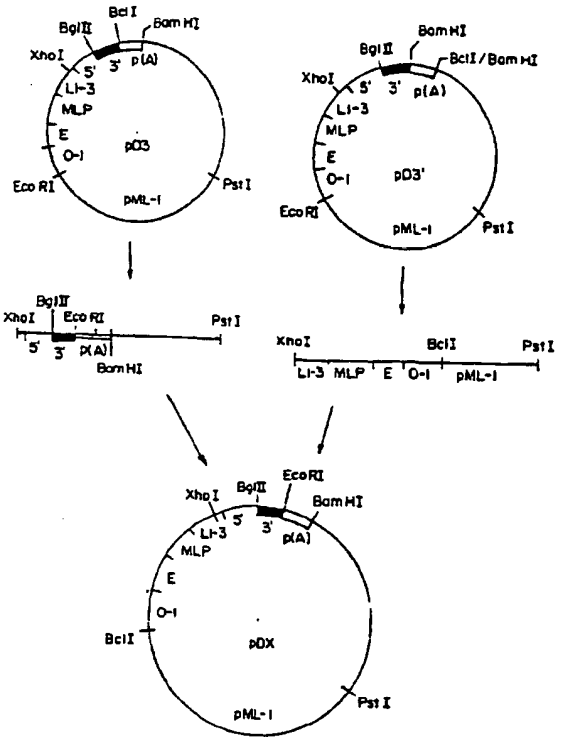
(81) Designated State: JP.

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*


(54) Title: ACTIVATED PROTEIN C WITH TRUNCATED LIGHT CHAIN


(57) Abstract

Proteins having the biological activity of human activated protein C are provided. The proteins are produced by mammalian host cells transfected with a plasmid capable of integration in mammalian host cell DNA. A method is also provided for activating plasma protein C or recombinant protein C, resulting in protein having substantially the activity of human activated protein C, wherein the light chain of the active protein contains 150, 151 or 152 amino acid residues. Compositions containing human activated protein $C^{150}$, activated protein $C^{151}$ and/or activated protein $C^{152}$ are also provided. A method for determining the ratio of these forms of protein C in purified activated protein C is also disclosed.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| **AT** | Austria | **ES** | Spain | **MG** | Madagascar |
| **AU** | Australia | **FI** | Finland | **ML** | Mali |
| **BB** | Barbados | **FR** | France | **MN** | Mongolia |
| **BE** | Belgium | **GA** | Gabon | **MR** | Mauritania |
| **BF** | Burkina Faso | **GB** | United Kingdom | **MW** | Malawi |
| **BG** | Bulgaria | **GN** | Guinea | **NL** | Netherlands |
| **BJ** | Benin | **GR** | Greece | **NO** | Norway |
| **BR** | Brazil | **HU** | Hungary | **PL** | Poland |
| **CA** | Canada | **IT** | Italy | **RO** | Romania |
| **CF** | Central African Republic | **JP** | Japan | **SD** | Sudan |
| **CG** | Congo | **KP** | Democratic People's Republic of Korea | **SE** | Sweden |
| **CH** | Switzerland | | | **SN** | Senegal |
| **CI** | Côte d'Ivoire | **KR** | Republic of Korea | **SU** | Soviet Union |
| **CM** | Cameroon | **LI** | Liechtenstein | **TD** | Chad |
| **CS** | Czechoslovakia | **LK** | Sri Lanka | **TG** | Togo |
| **DE** | Germany | **LU** | Luxembourg | **US** | United States of America |
| **DK** | Denmark | **MC** | Monaco | | |

## Description

# ACTIVATED PROTEIN C WITH TRUNCATED LIGHT CHAIN

## Technical Field

The present invention relates generally to plasma proteins and DNA sequences encoding those proteins and, more specifically, to the production of proteins having substantially the same biological activity as human activated protein C.

## Background of the Invention

Protein C is a zymogen, or precursor, of a serine protease that plays an important role in the regulation of blood coagulation and in the generation of fibrinolytic activity *in vivo*. It is synthesized in the liver as a single-chain polypeptide that undergoes considerable processing to give rise to a two-chain molecule comprising heavy (Mr = 40,000) and light (Mr = 21,000) chains held together by a disulfide bond. The circulating two-chain intermediate is converted to the biologically active form of the molecule, known as "activated protein C" (APC), by proteolytic processing, including removal of a 12-residue peptide (known as the activation peptide) from the amino-terminus of the heavy chain. The cleavage reaction is augmented *in vivo* by thrombomodulin, an endothelial cell co-factor (Esmon and Owen, Proc. Natl. Acad. Sci. USA 78:2249-2252, 1981).

Protein C is a vitamin K-dependent glycoprotein that contains approximately nine residues of gamma-carboxyglutamic acid (Gla) and one equivalent of beta-hydroxyaspartic acid, which are formed by post-translational modifications of glutamic acid and aspartic acid residues, respectively. The post-translational formation of specific gamma-carboxyglutamic acid residues in protein C requires vitamin K. These unusual amino acid residues bind to calcium ions and are believed to be responsible for the interaction of the protein with phospholipid, which is required for the biological activity of protein C.

In contrast to the coagulation-promoting action of other vitamin K-dependent plasma proteins, such as factor VII, factor IX, and factor X, activated protein C (APC) acts as a regulator of the coagulation process through the inactivation of factor Va and factor VIIIa by limited proteolysis. The inactivation of factors Va and VIIIa by protein C is dependent upon the presence of acidic phospholipids and calcium ions. Protein S has been reported to regulate this

activity by accelerating the APC-catalyzed proteolysis of factor Va (Walker, J. Biol. Chem. 255:5521-5524, 1980).

Protein C has also been implicated in the action of tissue-type plasminogen activator (Kisiel and Fujikawa, Behring Inst. Mitt. 73:29-42, 1983). Infusion of bovine APC into dogs results in increased plasminogen activator activity (Comp and Esmon, J. Clin. Invest. 68:1221-1228, 1981). Other studies (Sakata et al., Proc. Natl. Acad. Sci. USA 82:1121-1125, 1985) have shown that addition of APC to cultured endothelial cells leads to a rapid, dose-dependent increase in fibrinolytic activity in the conditioned media, reflecting increases in the activity of both urokinase-related and tissue-type plasminogen activators. APC treatment also results in a dose-dependent decrease in anti-activator activity. In addition, studies with monoclonal antibodies against endogenous APC (Snow et al., FASEB Abstracts, 1988) implicate APC in maintaining patency of arteries during fibrinolysis and limiting the extent of tissue infarct.

In some parts of the world, it is estimated that approximately 1 in 16,000 individuals exhibit protein C deficiency. Protein C deficiency is associated with recurrent thrombotic disease (Broekmans et al., New Eng. J. Med. 309:340-344, 1983, and Seligsohn et al., New Eng. J. Med. 310:559-562, 1984) and may result from genetic disorders or from trauma, such as injury, liver disease or surgery. Protein C deficiency is generally treated with oral anticoagulants. Beneficial effects have also been obtained through the infusion of protein C-containing normal plasma (see Gardiner and Griffin in Brown, Grune & Stratton (eds.), Prog. in Hematology 13:265-278, 1983, N.Y.). In addition, protein C is useful in treating thrombotic disorders, such as venous thrombosis (Smith et al., PCT Publication No. WO 85/00521).

Activated protein C may be preferred over the zymogen for the treatment of thrombosis. The use of activated protein C bypasses the need for *in vivo* activation of protein C, thus providing a faster-acting therapeutic agent. Several studies with baboon models of thrombosis have indicated that APC in low doses will be effective in the prevention of fibrin deposition, platelet deposition, and loss of circulation (Gruber et al., Hemostasis and Thrombosis 374a: abstract 1353, 1987; Widrow et al., Fibrinolysis 2 suppl. 1: abstract 7, 1988; Griffin et al., Thromb. Haemostasis 62: abstract 1512, 1989).

Finally, exogenous activated protein C has been shown to prevent the coagulopathic and lethal effects of gram negative septicemia (Taylor et al., J. Clin. Invest. 79:918-925, 1987). Data obtained from studies with baboons suggest that activated protein C plays a natural role in protecting against septicemia.

Protein C may be purified from clotting factor concentrates (Marlar et al., Blood 59:1067-1072, 1982) or from plasma (Kisiel, J. Clin. Invest. 64:761-769, 1979), although the process is complex, in part due to the limited availability of starting material and the low concentration of protein C in plasma. The therapeutic use of products derived from human blood carries the risk of disease transmission, and thus it is generally preferable to produce human protein C or activated protein C by genetic engineering techniques.

A cloned cDNA encoding most of human protein C is disclosed by Foster and Davie (Proc. Natl. Acad. Sci. USA 81:4766-4770, 1984). Foster et al. (Proc. Natl. Acad. Sci. USA 82:4673-4677, 1985) disclose the nucleotide sequence of the gene for human protein C and a tentative structure for human protein C. They suggest that the light chain of human protein C contains 155 amino acids, by analogy with the amino acid sequence of the light chain of bovine protein C reported by Fernlund and Stenflo (J. Biol. Chem. 257:12170-12179, 1982). Murray et al. (European Patent Office Publication 215,548) disclose methods for producing recombinant protein C and activated protein C. Bang et al. (U.S. Patent No. 4,775,624) disclose methods for producing recombinant protein C having an active light chain of 155 amino acids with the carboxyl-terminal sequence Met-Glu-Lys-Lys-Arg-Ser-His-Leu.

However, activated protein C may change its structure by exoproteolysis during storage, especially at a carboxy terminus of the light chain. This change in structure may influence its biological activity, which in turn may cause serious difficulties in the preparation of a pharmaceutical composition. Therefore, one object of the present invention is to provide a more stable APC for medical use.

## Summary of the Invention

Briefly stated, the present invention provides a more stable activated form of human protein C having a heavy chain and a light chain, the light chain consisting essentially of an amino acid sequence selected from the group consisting of the amino acid sequence of Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 150; the amino acid sequence of Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 151; and the amino acid sequence of Figure 1 from alanine, amino acid number 1, to arginine, amino acid number 152. In other embodiments, the activated human protein C is produced by activating recombinant protein C, by activating plasma protein C, or direct expression.

In another aspect of the present invention, a composition is provided comprising a first form of activated human protein C, a second form of activated human protein C, and/or a third form of activated human protein C, the first form comprising a heavy chain disulfide bonded to a light chain having the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 150; the second form comprising a heavy chain disulfide bonded to a light chain having the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 151, and a third form comprising a heavy chain disulfide bonded to a light chain having the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to arginine, amino acid number 152. In one embodiment of the composition, the ratio of the first form to the third form is between about 1:10 and 10:1. In another embodiment, the ratio is between 1:5 and 5:1. In yet another embodiment, the ratio is about 1:3. In still another embodiment of the composition, the ratio of the first form to the second form is between about 1:10 and 10:1, preferably between 1:5 and 5:1.

Those embodiments of the composition may contain a small portion of the remaining other form.

In another aspect of the present invention, the ratio in the above composition is determined by a method comprising the steps of (a) separating the light chains from the heavy chains; (b) fragmenting the separated light chains to produce polypeptides; (c) fractionating the polypeptides; (d) sequencing the fractionated polypeptides; and (e) calculating the molar ratio of the first form to the second form. In one embodiment, the separating step comprises reduction of disulfide bonds between the heavy chains and the light chains to produce -SH groups and modification of the -SH groups to prevent subsequent disulfidation. In another embodiment, the fragmenting step comprises proteolysis with endoproteinase Asp-N, or proteolysis with endoproteinase Asp-N, optionally followed by proteolysis with chymotrypsin after separation of a peptide. In yet another embodiment, the fractionating step comprises high performance liquid chromatography.

In another aspect of the present invention, a method is provided for producing a composition comprising human activated protein $C^{150}$, human activated protein $C^{151}$, and/or human activated protein $C^{152}$ from human protein C by the process of (a) concentrating the human protein C; (b) reducing the salt concentration of the concentrated protein C to produce a reduced salt solution; (c) exposing the reduced salt solution to thrombin to activate the protein C;

(d) purifying the activated protein C from the thrombin by subjecting the product of step (c) to ion exchange chromatography to produce one or more fractions containing purified activated protein C; and (e) collecting the fraction(s) containing the purified activated protein C, wherein the purified activated protein C consists essentially of activated protein $C^{150}$, activated protein $C^{151}$, and/or activated protein $C^{152}$. In another embodiment, the composition comprises a ratio of activated protein $C^{150}$ to activated protein $C^{152}$ is between about 1:10 and 10:1. In yet another embodiment, the ratio is about 1:5 and 5:1. In a further embodiment, the ratio is about 1:3. In another embodiment, the composition comprises a ratio of activated protein $C^{150}$ to activated protein $C^{151}$ between about 1:10 and 10:1. In yet another embodiment, the ratio is between about 1:5 and 5:1. In a further embodiment, the ratio is about 3:1. In a further embodiment, the step of exposing comprises combining the reduced salt solution with thrombin to produce a ratio of protein C to thrombin of from about 1:1 to about 200:1 by weight. In another embodiment, the ratio is approximately 20:1. In yet another embodiment, the human protein C is concentrated to between about 2.0 to 2.5 mg/ml. In another embodiment, the step of purifying comprises cation exchange chromatography followed by anion exchange chromatography. In another embodiment, the human protein C is recombinant protein C which is produced by culturing mammalian cells transfected to express a protein C precursor having the sequence $R_1$-$R_2$-$R_3$-$R_4$ at the junction between the light chain and the activation peptide, wherein each of $R_1$ through $R_4$ is a lysine residue or an arginine residue.

In another aspect of the present invention, a pharmaceutical composition is provided containing a physiologically acceptable carrier or diluent and activated plasma protein C or activated recombinant protein C, wherein the activated plasma or recombinant protein C has a heavy chain and a light chain, the light chain consisting essentially of the amino acid sequence of Figure 1 from alanine, amino acid number 1, to an amino acid selected from the group consisting of lysine, amino acid number 150, lysine, amino acid number 151, and arginine, amino acid number 152. In one embodiment, the pharmaceutical composition contains a ratio of activated protein $C^{150}$ to activated protein $C^{152}$ of about 1:10 to 10:1. In another embodiment, the ratio is about 1:5 to 5:1. In yet another embodiment, the ratio is 1:3.

In another embodiment, the pharmaceutical composition contains a ratio of activated protein $C^{150}$ to activated protein $C^{151}$ of between about 1:10

and 10:1. In a further embodiment, the ratio is about 1:5 to 5:1. In yet another embodiment, the ratio is about 3:1.

In yet another aspect of the present invention, a method is provided for determining the ratio between activated protein $C^{150}$, activated protein $C^{151}$ and activated protein $C^{152}$ in purified activated protein C, comprising the steps of (a) reducing a disulfide bond which connects the heavy chain and the light chain of activated protein C to an -SH group; (b) modifying the -SH group such that further disulfidation is prevented; (c) separating the light chain from the heavy chain; (d) fragmenting the light chain; (e) fractionating the fragmented light chain; (f) sequencing the fractionated light chain; and (g) calculating the molar ratio between activated protein $C^{150}$, activated protein $C^{151}$, and activated protein $C^{152}$.

These and other aspects will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 illustrates the nucleotide sequence of the complete protein C cDNA and the deduced amino acid sequence of human protein C. The arrow indicates the junction of the activation peptide and the heavy chain. The heavy chain of activated protein C extends from amino acid number 170, leucine, to amino acid number 419, proline.

Figure 2 illustrates the construction of the vector pD3. Symbols used are 0-1, the adenovirus 5 0-1 map unit sequence; E, the SV40 enhancer; MLP, the adenovirus 2 major late promoter; L1-3, the adenovirus 2 tripartite leader; 5', 5' splice site; 3', 3' splice site; p(A), polyadenylation signal; and DHFR, dihydrofolate reductase gene.

Figure 3 illustrates the construction of the vector pDX. Symbols are used as set forth Figure 2.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Biological Activity: A function or set of functions performed by a molecule in a biological context (i.e., in an organism or an *in vitro* facsimile thereof). Biological activities of proteins may be divided into catalytic and effector activities. Catalytic activities of vitamin K-dependent plasma proteins generally involve specific proteolytic cleavages of other plasma proteins, resulting in

activation or deactivation of the substrates. Effector activities include specific binding of the biologically active molecule to calcium, phospholipids or other small molecules, to macromolecules, such as proteins, or to cells. Effector activity frequently augments, or is essential to, catalytic activity under physiological conditions.

For activated protein C, biological activity is characterized by its anticoagulant and fibrinolytic properties. Activated protein C inactivates factor Va and factor VIIIa in the presence of acidic phospholipids and calcium. Protein S appears to be involved in the regulation of this function (Walker, ibid.). Activated protein C also enhances fibrinolysis, an effect believed to be mediated by the lowering of plasminogen activator inhibitor levels (van Hinsbergh et al., Blood 65:444-451, 1985). The catalytic activities of activated protein C reside in the heavy chain. A protein having substantially the same biological activity as protein C will be essentially free of this activity until activated.

Activated Protein C: A protein having the activity of activated protein C as defined above. The protein will include a catalytic heavy chain disulfide bonded to an effector light chain containing a calcium binding gla domain. The light chain may be the 150, 151, or 152 amino acid light chain of native human protein C, or may include amino acid substitutions that do not substantially alter its effector activities. Activated protein $C^{150}$ ($APC^{150}$) refers to activated protein C wherein the light chain has the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 150. Activated protein $C^{151}$ ($APC^{151}$) refers to activated protein C wherein the light chain has the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 151. Activated protein $C^{152}$ ($APC^{152}$) refers to activated protein C wherein the light chain has the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to arginine, amino acid number 152.

Pre-Pro Peptide: An amino acid sequence that occurs at the amino terminus of some proteins and is generally cleaved from the protein during translocation through the secretory pathway. Pre-pro peptides comprise sequences directing the protein into the secretory pathway of the cell (signal peptides) that are characterized by the presence of a core of hydrophobic amino acids. Pre-pro peptides may also comprise processing signals. As used herein, the term "pre-pro peptide" may also mean a functional portion of a naturally occurring pre-pro peptide.

Expression Vector: A DNA molecule which contains, *inter alia*, a DNA sequence encoding a protein of interest together with a promoter and other sequences, such as a transcription terminator and polyadenylation signal, that facilitate expression of the protein. Expression vectors further contain genetic information that provides for their replication in a host cell, either by autonomous replication or by integration into the host genome. Examples of expression vectors commonly used for recombinant DNA are plasmids and certain viruses, although they may contain elements of both. They also may include a selectable marker.

Cultured Mammalian Cells: Cells that have been isolated from a mammal and are able to be propagated *in vitro*.

DNA Construct: A DNA molecule, or a clone of such a molecule, which has been constructed through human intervention to contain sequences arranged in a way that would not otherwise occur in nature.

As noted above, protein C is initially produced as a single-chain polypeptide which undergoes extensive processing to yield activated protein C. This processing includes the formation of specific gamma-carboxyglutamic acid residues in the amino-terminal region of the light chain, beta-hydroxylation of an aspartic acid residue and proteolytic cleavage.

Also as noted above, human activated protein C is generally believed to contain a light chain consisting of 155 amino acid residues (Foster et al., ibid.; Bang et al., ibid.). However, the inventors have found that activated protein C is heterogeneous and contains biologically active species consisting of 150, 151, and 152 amino acid residues. Based on this discovery, the inventors have developed processes for producing recombinant and plasma-derived protein C which, upon activation, direct expression, and purification, is recoverable as a defined, homogeneous composition. Activated protein C produced according to the present invention will have a light chain of 150, 151, or 152 amino acids, or will be a mixture of the 150, 151, and/or 152 amino acid light chain forms.

Cloned DNA sequences encoding protein C have been described (Foster and Davie, Proc. Natl. Acad. Sci. USA 81:4766-4770, 1984; Foster et al., Proc. Natl. Acad. Sci. USA 82:4673-4677, 1985; and Bang et al., U.S. Patent No. 4,775,624). In general, cDNA sequences are preferred for producing recombinant protein C due to their lack of intervening sequences. Complementary DNAs encoding protein C may be obtained from libraries prepared from liver cells according to standard laboratory procedures. It will be understood, however, that suitable DNA sequences can also be obtained from genomic clones or can be

synthesized *de novo* according to conventional procedures. Techniques for producing synthetic nucleotide sequences are well known in the art. For example, a set of overlapping oligonucleotides may be synthesized and annealed in pairs to yield double-stranded fragments with overlapping adhesive termini. These fragments are then ligated as necessary to provide a complete coding sequence. When using genomic sequences, it is generally desirable to remove introns. If partial clones are obtained, it is necessary to join them in proper reading frame to produce a full length clone, using such techniques as endonuclease cleavage, ligation, and loop-out mutagenesis.

The coding sequence for protein C will encode a pre-pro peptide at the amino-terminus of the protein in order to obtain proper post-translational processing (e.g., gamma-carboxylation of glutamic acid residues) and secretion from the host cell. The pre-pro peptide may be that of protein C or another vitamin K-dependent plasma protein, such as factor VII, factor IX, factor X, prothrombin or protein S.

The amino-terminal portion (gla domain) of recombinant protein C may be substituted with the gla domain of a vitamin-K dependent plasma protein selected from the group consisting of factor VII, factor IX, factor X, prothrombin or protein S. The amino-terminal portions of vitamin K-dependent plasma proteins are responsible for at least part of their respective calcium binding activities. It has been found that, as a result of this functional homology, the gla domains of these molecules may be interchanged and the resulting chimeric proteins still retain the activity specific to the catalytic domain. For example, as described in U.S. Patent No. 4,789,950, the amino-terminal gla domain of factor IX may be joined to factor VII at amino acid 38 to produce a protein having the activity of factor VII. Factor VII, factor IX, factor X, prothrombin, and protein S share this amino-terminal sequence homology with protein C. This region of homology spans approximately 35-45 amino acid residues, with a C-terminal boundary generally corresponding to an exon-intron boundary in the respective gene. The gla domain of human protein C extends from amino acid number 1 of the mature light chain to approximately amino acid number 37 as shown in Figure 1. Thus, a cloned sequence comprising the 5'-coding region of the gene for any of these proteins may be substituted for the corresponding sequence of the protein C gene. The resultant activated protein C precursor will comprise a hybrid light chain, including a gla domain operably joined to the gla domain-less light chain of protein C. It is preferred that the pre-pro sequence and gla domain of such protein C precursors be derived from the same protein.

The proteins of the present invention can be produced from recombinant protein C or plasma protein C. Methods for producing recombinant and plasma protein C are known in the art.

The cloned DNA sequence may be further modified to facilitate processing of the encoded protein C precursor. A preferred modification in this regard is the alteration of the junction point between the light chain and the activation peptide to include the sequence $R_1$-$R_2$-$R_3$-$R_4$, wherein each of $R_1$ through $R_4$ is a lysine residue or an arginine residue. A preferred such sequence is Arg-Arg-Lys-Arg. One such modified protein C precursor has the amino acid sequence: light chain (155 amino acids)-Arg-Arg-Lys-Arg-Asn-Ile-Leu-Asn-Arg-Arg-Lys-Arg-heavy chain. Modification may be achieved by site-specific mutagenesis. Techniques of site-specific mutagenesis are well known in the art and are described by, for example, Zoller and Smith (DNA 3:479-488, 1984). Alternatively, the protein C sequence may be enzymatically cleaved to remove nucleotides around the junction point, and the sequences encoding the heavy and native light chains joined to a synthesized linker encoding an altered junction sequence.

DNA sequences useful in carrying out the present invention also include those coding for the direct expression of APC. APC precursor sequences may be co-expressed with the *Saccharomyces cerevisiae KEX2* gene in a host cell as described in U.S. Patent Application Serial No. 07/130,370 and European Patent Office publication EP 319,944.

The DNA sequence encoding protein C or an activated protein C precursor is inserted into a suitable expression vector, which is in turn used to transfect cultured mammalian cells. Expression vectors for use in carrying out the present invention will comprise a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include both viral promoters and cellular promoters. Viral promoters useful in this regard include the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1:854-864, 1981) and the CMV promoter (Boshart et al., Cell 41:521-530, 1985). A particularly preferred viral promoter is the major late promoter from adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2:1304-1319, 1982). Cellular promoters include the mouse kappa gene promoter (Bergman et al., Proc. Natl. Acad. Sci. USA 81:7041-7045, 1983) and the mouse $V_H$ promoter (Loh et al., Cell 33:85-93, 1983). A particularly preferred cellular promoter is the metallothionein I promoter (Palmiter et al., Science 222:809-814, 1983; Palmiter et al., U.S. Patent No. 4,579,821). Expression vectors may also contain a set of RNA splice sites located downstream from the promoter

and upstream from the insertion site for the protein C sequence or within the protein C sequence itself. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also generally contained in the expression vectors are transcription termination and polyadenylation signals located downstream of the insertion site. Particularly preferred polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the adenovirus 5 Elb region, the human growth hormone gene terminator (DeNoto et al., Nuc. Acids Res. 9:3719-3730, 1981) and the protein C gene polyadenylation signal. The expression vectors may also include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites, and enhancer sequences, such as the SV40 enhancer and the sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences are then introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725-732, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603-616, 1981; Graham and Van der Eb, Virology 52:456-467, 1973) or electroporation (Neumann et al., EMBO J. 1:841-845, 1982). In order to identify cells that have integrated the DNA, a gene that confers a selectable phenotype (a selectable marker) is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers include genes that confer resistance to drugs such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is a gene encoding dihydrofolate reductase (DHFR), which confers resistance to the drug methotrexate. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, Mass.), and the choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339 and U.S. Patent Application Serial No. 07/226,173). It may also be advantageous to add additional DNA, known as "carrier DNA," to the mixture that is introduced into the cells.

After the cells have taken up the DNA, they are cultured to produce protein C or activated protein C. The cells are cultured according to standard methods in a growth medium containing nutrients required for growth of mammalian cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The medium may contain serum, such as bovine serum, or may be serum-free. For production of biologically active protein C, the medium will generally also contain vitamin K at a concentration of about 0.1 μg/ml to about 5 μg/ml. Drug selection is then applied to select for the growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker, the drug concentration may be increased to select for an increased copy number of the cloned sequences, thereby increasing expression levels. Clones of stably transfected cells are then screened for expression of protein C.

Preferred cultured mammalian cells for use in the present invention include the COS-1 (ATCC CRL 1650), BHK and 293 (ATCC CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk$^-$ ts13 BHK cell line (Waechter and Baserga, Proc. Natl. Acad. Sci. USA 79:1106-1110, 1982), hereinafter referred to as "BHK 570" cells. The BHK 570 cell line has been deposited with the American Type Culture Collection, Rockville, Md., under accession number CRL 10314. A tk$^-$ts13 BHK cell line is also available from American Type Culture Collection under accession number CRL 1632. In addition, a number of other cell lines may be used within the present invention, including RAT Hep I (ATCC CRL 1600), RAT Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1), CHO (ATCC CCL 61), and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216-4220, 1980).

Recombinant protein C is then isolated by removing the conditioned culture media from the cells, then treating the media to remove remaining cells and cell debris, such as by centrifugation or filtration. It is preferred that the protein C-containing media then be concentrated to facilitate subsequent purification and handling. Methods for concentrating protein solutions are known in the art, a preferred method being ultrafiltration. The conditioned media will typically be concentrated about 20- to 40-fold. The concentrated media are then treated to reduce the salt concentration, such as by dialysis against a low-salt buffer at approximately physiological pH. A preferred

such buffer is 0.05 M Tris HCl, pH 7.4 containing 0.15 M NaCl. The protein C is then purified from the concentrated conditioned media. Methods for purifying protein C are known in the art, and may include such steps as immunoaffinity chromatography, precipitation with barium citrate and high performance liquid chromatography. A preferred method of purification is affinity chromatography on an anti-protein C antibody column. The use of calcium-dependent monoclonal antibodies, as described by Wakabayashi et al. (J. Biol. Chem. 261:11097-11108, 1986), is particularly preferred. The conditioned medium is applied to the column in the presence of a low concentration of $Ca^{++}$, preferably by adjusting the composition of the medium to about 5 mM $CaCl_2$. The protein C is then eluted from the column using a chelating agent, such as 10 mM EDTA. Column fractions are collected, and the protein C-containing fractions are pooled. The protein C purity of the column eluate fractions may be determined by polyacrylamide gel electrophoresis.

Alternatively, protein C may be isolated from human plasma according to standard procedures (e.g., Kisiel et al., J. Clin. Invest. 64:761, 1979; Kisiel and Davie, Meth. Enzymol. 80:320-332, 1981), or may be obtained from commercial sources.

If produced in the zymogen form, the purified protein C is then activated. The protein is concentrated, preferably by ultrafiltration, to a concentration of about 0.06 to 6 mg/ml, preferably about 2.0 to 2.5 mg/ml. The salt concentration in the concentrated protein solution is then reduced, such as by dialyzing it against a low ionic strength buffer of approximately physiological pH. A preferred buffer is 0.05 M Tris HCl, pH 7.5 containing 0.15 M NaCl. Activation is achieved by combining the protein C solution with thrombin, preferably purified bovine $\alpha$-thrombin, containing less than 10%, more preferably less than 5%, bovine $\beta$- or $\gamma$-thrombin, at a weight ratio of about 1:1 to about 200:1 protein C to thrombin, preferably about 20:1 protein C to thrombin. The solution is mixed and incubated at a temperature between about 4°C and about 37°C for 0.5 to 24 hours, preferably about 3 to 5 hours.

The activated protein C is then purified from the activation mixture by ion exchange chromatography. It is preferred to use a combination of cation exchange chromatography and anion exchange chromatography.

In a preferred embodiment, the activation mixture is diluted and applied to a cation exchange column. Suitable cation exchange media include CM ion exchange gels and SP ion exchange gels. A particularly preferred cation exchange medium is S-Sepharose (Pharmacia, Piscataway, N.J.). The column is

equilibrated with a low-ionic strength, slightly acidic buffer, such as 0.02 M MES/Tris pH 6.0, 0.05 M NaCl. The activated protein C is eluted from the column using a salt gradient. APC will elute in the pass-through fractions and at a salt concentration of about 0.3 to 0.5 M NaCl. Thrombin will elute from the column at about 0.6 M NaCl. The APC-containing fractions are pooled, and the salt concentration is reduced to not more than about 0.2 M NaCl, such as by dilution with a low-salt buffer. The resulting low-salt solution is applied to an anion exchange column. Suitable anion exchange media include DEAE ion exchange gels and QAE ion exchange gels. A particularly preferred medium is Q-Sepharose (Pharmacia, Piscataway, N.J.). The APC-containing solution is applied to the column, which is previously equilibrated with a slightly acidic, low-ionic strength buffer. The APC is eluted with a salt gradient or by washing the column with buffer containing about 0.3 to 0.5 M NaCl, preferably about 0.4 M NaCl. The presence of APC in the column fractions may be determined throughout the purification procedure by monitoring the absorbance of the solution at 280 nm and by polyacrylamide gel electrophoresis.

If necessary, additional purification may be achieved by affinity chromatography, preferably immunoaffinity chromatography. For example, contaminating zymogen protein C may be removed from the APC preparation by immunoaffinity chromatography on an antibody specific for the activation peptide of protein C. Additional purification may also be achieved by immunoaffinity chromatography using an antibody specific for the gla domain of protein C.

The carboxy-terminus of activated protein C (APC) may be determined by isolating C-terminal light chain peptides and sequencing them by methods known in the art. Generally, the heavy chain and the light chain of APC are first separated by cleaving the S-S bond which connects them. There are many methods well known in the art for cleaving S-S bonds, such as the use of dithiothreitol in a 6 M guanidine-HCl solution at pH 8.3. Cleaved products are preferably treated with monoiodoacetic acid to prevent re-disulfidation.

The mixed heavy- and light-chain proteins may then be fractionated, for instance, by reversed-phase partition chromatography. In a preferred embodiment, a Poly-F column (Du Pont) is used.

The isolated light chain is then treated to cleave it, and the resulting peptides are sequenced. Methods of treatment include proteolysis by cyanogen bromide, or enzymatic proteolysis, such as proteolysis using endoproteinase Asp-N.

The mixture of peptide fragments of the light chain may be further separated into peptide fractions. A preferred method is reversed-phase partition chromatography with an octadecyl silanol (ODS) column. Peptide fractions may then be sequenced by any of the methods well known in the art. Preferably, an amino acid sequencer is used. Peptides having an amino-terminus at amino acid No. 128 are generally present among the group of peaks.

Based on the results of the amino acid sequence analysis, the amount (pmol) of light chain peptide 128-150 is determined by subtracting the amount of other peptides contained in the same peak. The amount of peptide is determined from the molar ratio of each amino acid in the earliest cycle in which amino acids showing the same degree of recovery are detected. The amount of light chain peptide 128-152 is determined in the same manner. The molar ratio of peptide 128-150 to peptide 128-152 is considered to be the ratio of $APC^{150}/APC^{151}/APC^{152}$.

$APC^{150}$, $APC^{151}$ and $APC^{152}$ may be separated from each other on the basis of the charge difference imparted by the two additional basic amino acids present on $APC^{152}$. Protein C is anionic at neutral pH, with a pI of approximately 4.5, facilitating the separation of the two forms by anion exchange chromatography. Preferred chromatographic media in this regard include DEAE resins, such as DEAE Sephadex (Pharmacia, Piscataway, N.J.). Briefly, a solution containing the two forms of protein C is applied to an anion exchange column at about pH 6.5. The proteins are sequentially eluted with a salt gradient such as a gradient of about 0-1 M NaCl, whereby $APC^{152}$ elutes at a lower salt concentration than $APC^{150}$. Fractions are collected and monitored for the presence of protein by standard methods, such as absorbance of the solution at 280 nm. In a similar manner, $APC^{150}$ is isolated from mixtures containing longer or shorter light chain species on the basis of the charge difference imparted by the single C-terminal basic amino acid present on its light chain.

The peptide obtained by treating with the endoproteinase Asp-N can further be treated with chymotrypsin and analyzed in the same manner described above. Analysis using this method for an activated protein C derived from a plasma protein C showed mainly $APC^{150}$ and $APC^{151}$ and a small amount of $APC^{152}$. This analysis can be confirmed with amino acid analysis.

The activated protein C of the present invention may be used in pharmaceutical compositions for topical or intravenous administration, generally in combination with a physiologically acceptable carrier or diluent. Preferred carriers and diluents include water, buffered water, 0.4% saline, 0.3% glycine and

the like. Pharmaceutical compositions may also contain stabilizers and adjuvants. These compositions may be sterilized by conventional, well known sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may further contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, acetate, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium citrate, amino acetate, etc., as well as additional therapeutic ingredients, such as plasminogen activators, heparin or antithrombin III. The concentration of protein C in these formulations can vary widely, i.e., from less than about 0.5% to as much as 15% to 20% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250~1000 ml of sterile Ringer's solution and 1~10 mg of protein C. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 16th ed., Mack Publishing Company, Easton, Pa. (1982), which is incorporated herein by reference.

The pharmaceutical compositions containing protein C can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, the compositions are administered in an amount sufficient to cure or at least partially arrest the disease and its complications. Amounts effective for this use will depend on the severity of the disease or injury and the general state of the patient, but generally range from about 0.1 mg to about 300 mg of protein C per day, with dosages of from about 1 mg to about 25 mg of protein C per day being more commonly used. It must be kept in mind that the materials of the present invention may generally be employed in serious disease or injury states, that is, life-threatening or potentially life-threatening situations. In such cases, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these protein C compositions.

In prophylactic applications, compositions containing the protein C are administered to a patient susceptible to or otherwise at risk of a disease state or injury to enhance the patient's own anticoagulative or fibrinolytic capabilities. In this use, the precise amounts again depend on the patient's state of health and

general level of endogenous protein C, but generally range from about 0.5 mg to about 250 mg per 70 kilogram patient, especially about 1 mg to about 25 mg per 70 kg of body weight.

The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

Restriction endonucleases and other DNA modification enzymes (e.g., T4 polynucleotide kinase, calf alkaline phosphatase, DNA polymerase I [Klenow fragment], T4 polynucleotide ligase) were obtained from Bethesda Research Laboratories (BRL) and New England Biolabs and were used as directed by the manufacturer, unless otherwise noted.

Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis on denaturing gels. *E. coli* cells were transformed as described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). M13 and pUC cloning vectors and host strains were obtained from BRL.

## EXAMPLE 1

### Cloning of DNA Sequences Encoding Human Protein C

A cDNA coding for a portion of human protein C was prepared as described by Foster and Davie (ibid.) Briefly, a λgt11 cDNA library was prepared from human liver mRNA by conventional methods. Clones were screened using an $^{125}$I-labeled affinity-purified antibody to human protein C, and phage were prepared from positive clones by the plate lysate method (Maniatis et al., ibid.), followed by banding on a cesium chloride gradient. The cDNA inserts were removed using Eco RI and subcloned into plasmid pUC9 (Vieira and Messing, Gene 19:259-268, 1982). Restriction fragments were subcloned in the phage vectors M13mp10 and M13mp11 (Messing, Meth. Enzymol. 101:20-77, 1983) and sequenced by the dideoxy method (Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977). A clone was selected that contained DNA corresponding to the known partial sequence of human protein C (Kisiel, ibid., 1979) and encoded protein C beginning at amino acid 64 of the light chain and extending through the heavy chain and into the 3' non-coding region. This clone was designated λHC1375. A second cDNA clone coding for protein C from amino acid 24 was also identified. The insert from the larger clone was subcloned into pUC9 and the plasmid was designated pHCλ6L. This clone encodes a major portion of

protein C, including the heavy chain coding region, termination codon, and 3' non-coding region.

The cDNA insert from λHC1375 was nick translated using α-$^{32}$P dNTP's and used to probe a human genomic library in phage λCharon 4A (Maniatis et al., Cell 15:687-702, 1978) using the plaque hybridization procedure of Benton and Davis (Science 196:181-182, 1977) as modified by Woo (Meth. Enzymol. 68:381-395, 1979). Positive clones were isolated and plaque-purified (Foster et al., Proc. Natl. Acad. Sci. USA 82:4673-4677, 1985, herein incorporated by reference). Phage DNA prepared from positive clones (Silhavy et al., in Experiments with Gene Fusion, Cold Spring Harbor Laboratory, 1984) was digested with Eco RI or Bgl II and the genomic inserts were purified and subcloned in pUC9. Restriction fragments of the genomic inserts were subcloned into M13 vectors and sequenced to confirm their identity and establish the DNA sequence of the entire gene.

The cDNA insert of pHCλ6L was nick translated and used to probe the phage λCharon 4A library. One genomic clone was identified that hybridized to probes made from the 5' and 3' ends of the cDNA. This phage clone was digested with Eco RI, and a 4.4 kb fragment, corresponding to the 5' end of the protein C gene, was subcloned into pUC9. The resultant recombinant plasmid was designated pHCR4.4. Complete DNA sequence analysis revealed that the insert in pHCR4.4 comprised two exons of 70 and 167 base pairs separated by an intron of 1263 bp. The first exon encodes amino acids -42 to -19; the second encodes amino acids -19 to 37. Sequence analysis confirmed the DNA sequence of the entire protein C gene.

A genomic fragment containing an exon corresponding to amino acids -42 and -19 of the pre-pro peptide of protein C was isolated, nick translated, and used as a probe to screen a cDNA library constructed by the technique of Gubler and Hoffman (Gene 25:263-269, 1983) using mRNA from Hep G2 cells. This cell line was derived from human hepatocytes and was previously shown to synthesize protein C (Fair and Bahnak, Blood 64:194-204, 1984). Ten positive clones comprising cDNA inserted into the Eco RI site of phage λgt11 were isolated and screened with an oligonucleotide probe corresponding to the 5' non-coding region of the protein C gene. One clone was also positive with this probe and its entire nucleotide sequence was determined. The cDNA contained 70 bp of 5' untranslated sequence, the entire coding sequence for human pre-pro-protein C, and the entire 3' non-coding region corresponding to the second polyadenylation site (Figure 1).

## EXAMPLE 2

### Expression of Protein C

A. Construction of Vector pDX

The vector pDX was derived from pDHFRIII (Berkner and Sharp, Nuc. Acids Res. 13:841-857, 1985) as shown in Figures 2 and 3.

The Pst I site immediately upstream from the DHFR sequence in pDHFRIII was converted to a Bcl I site by digesting 10 μg of plasmid with 5 units of Pst I for 10 minutes at 37°C in 100 μl restriction buffer A (10 mM Tris pH 8, 10 mM $MgCl_2$, 6 mM NaCl, 7 mM β-MSH). The DNA was phenol extracted, ethanol precipitated, and resuspended in 40 μl polymerase buffer (50 mM Tris pH 8, 7 mM $MgCl_2$, 7 mM β-MSH) containing 10 mM dCTP and 16 units T4 DNA polymerase and incubated at 12°C for 60 minutes. Following ethanol (EtOH) precipitation, the DNA was ligated to 2.5 μg kinased Bcl I linkers in 14 μl ligase buffer (10 mM Tris pH 8, 10 mM $MgCl_2$, 1 mM DTT, 1.4 mM ATP) containing 400 units T4 polynucleotide ligase for 12 hours at 12°C. Following phenol extraction and EtOH precipitation, the DNA was resuspended in 120 μl restriction buffer B (75 mM KCl, 6 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT), digested with 80 units Bcl I for 60 minutes at 50°C, then electrophoresed through agarose. Form III plasmid DNA (10 μg) was isolated from the gel and ligated in 10 μl buffer C containing 50 units T4 polynucleotide ligase for two hours at 12°C, then used to transform *E. coli* HB101. Positive colonies were identified by rapid DNA preparation analysis, and plasmid DNA (designated pDHFR') prepared from positive colonies was transformed into dam⁻ *E. coli*.

Plasmid pD2' was then generated by cleaving pDHFR' (15 μg) and pSV40 (comprising Bam HI digested SV40 DNA cloned into the Bam HI site of pML-1) (25 μg) in 100 μl restriction buffer B with 25 units Bcl I for 60 minutes at 50°C, followed by the addition of 50 units of Bam HI and additional incubation at 37°C for 60 minutes. DNA fragments were resolved by agarose gel electrophoresis, and the 4.9 kb pDHFR' fragment and 0.2 kb SV40 fragment were isolated. These fragments (200 ng pDHFR' DNA and 100 ng SV40 DNA) were incubated in 10 μl ligase buffer containing 100 units T4 polynucleotide ligase for 4 hours at 12°C, and the resulting construct (pD2') was used to transform *E. coli* RR1.

Plasmid pD2' was modified by deleting the "poison" sequences in the pBR322 region (Lusky and Botchan, Nature 293:79-81, 1981). Plasmids pD2' (6.6 μg) and pML-1 (Lusky and Botchan, ibid.) (4 μg) were incubated in 50 μl restriction buffer A with 10 units each Eco RI and Nru I for two hours at 37°C, followed

by agarose gel electrophoresis. The 1.7 kb pD2' fragment and the 1.8 kb pML-1 fragment were isolated and ligated together (50 ng each) in 20 µl ligase buffer containing 100 units T4 polynucleotide ligase for two hours at 12°C, followed by transformation into *E. coli* HB101. Colonies containing the desired construct (designated pD2) were identified by rapid preparation analysis. Ten µg of pD2 was then digested with 20 units each Eco RI and Bgl II in 50 µl restriction buffer A for two hours at 37°C. The DNA was electrophoresed through agarose, and the desired 2.8 kb fragment, comprising the pML-1, 3' splice site and poly (A) sequences, was isolated.

Plasmid pDHFRIII was modified to convert the Sac II (Sst II) site into either a Hind III or Kpn I site. Ten µg pDHFRIII was digested with 20 units Sst II for two hours at 37°C, followed by phenol extraction and ethanol precipitation. Resuspended DNA was incubated in 100 µl polymerase buffer containing 10 mM dCTP and 16 units T4 DNA polymerase for 60 minutes at 12°C, phenol extracted, dialyzed, and ethanol precipitated. DNA (5 µg) was ligated with 50 ng kinased Hind III or Kpn I linkers in 20 µl buffer C containing 400 units T4 ligase for 10 hours at 12°C, phenol extracted, and ethanol precipitated. After resuspension in 50 µl restriction buffer A, the resultant plasmids were digested with 50 units Hind III or Kpn I, as appropriate, and electrophoresed through agarose. Gel-isolated DNA (250 ng) was ligated in 30 µl ligase buffer containing 400 units T4 DNA ligase for 4 hours at 12°C and used to transform *E. coli* RR1. The resultant plasmids were designated pDHFRIII (Hind III) and pDHFRIII (Kpn I). A 700 bp Kpn I-Bgl II fragment was then purified from pDHFRIII (Kpn I) by digestion with Bgl II and Kpn I followed by agarose gel electrophoresis.

The SV40 enhancer sequence was inserted into pDHFRIII (Hind III). Fifty µg SV40 DNA was incubated in 120 µl restriction buffer A with 50 units Hind III for 2 hours at 37°C, and the Hind III SV40 fragment (5089-968 bp) was gel purified. Plasmid pDHFRIII (Hind III) (10 µg) was treated with 250 ng calf intestinal phosphatase for one hour at 37°C, phenol extracted and ethanol precipitated. The linearized plasmid (50 ng) was ligated with 250 ng of the SV40-Hind III fragment in 16 µl ligase buffer for 3 hours at 12°C, using 200 units T4 polynucleotide ligase, and transformed into *E. coli* HB101. A 700 base pair Eco RI-Kpn I fragment was then isolated from this plasmid.

Plasmid pD3 was then constructed. The 700 bp Kpn I-Bgl II fragment and the 700 bp Eco RI-Kpn I fragment (50 ng each) were ligated with 10 ng of the 2.8 kb pML-1, 3' splice site, poly(A) fragment with 200 units T4 polynucleotide ligase for 4 hours at 12°C, followed by transformation of *E. coli*

RR1. Positive colonies were detected by rapid preparation analysis, and a large-scale preparation of pD3 (Figure 2) was made.

The vector pD3' was constructed in a similar manner, except that the SV40 polyadenylation signal (i.e., the SV40 Bam HI [2533 bp] to Bcl I [2770 bp] fragment was inserted in the late orientation. Thus, pD3' contains a Bam HI site as the site of gene insertion (Figure 3).

The vector pDX was then generated from pD3 and pD3' as shown in Figure 3. The Eco RI site in pD3' was converted to a Bcl I site by Eco RI cleavage, incubation with S1 nuclease, and subsequent ligation with Bcl I linkers. DNA was prepared from a positively identified colony, and the 1.9 kb Xho I-Pst I fragment containing the altered restriction site was prepared via agarose gel electrophoresis. In a second modification, Bcl I-cleaved pD3 was ligated with kinased Eco RI-Bcl I adapters (constructed from oligonucleotides ZC525, 5'GGA ATT CT 3'; and ZC526, 5'GAT CAG AAT TCC 3') in order to generate a unique Eco RI site for inserting a gene into the expression vector. A positive colony was identified by restriction endonuclease analysis, and DNA from this colony was used to isolate a 2.3 kb Xho I-Pst I fragment containing the modified restriction site. The two above-described DNA fragments were incubated together with T4 DNA ligase, transformed into *E. coli* HB101, and positive colonies were identified by restriction analysis. Plasmid DNA was isolated and designated pDX (Figure 3). This plasmid contains a unique Eco RI site for insertion of foreign genes.

B. cDNA Expression

The protein C cDNA was inserted into pDX as an Eco RI fragment. Recombinant plasmids were screened by restriction analysis to identify those having the protein C insert in the correct orientation with respect to the promoter elements, and plasmid DNA (designated pDX/PC) was prepared from a correct clone.

Because the cDNA insert in pDX/PC contains an ATG codon in the 5' non-coding region (see Figure 1), deletion mutagenesis was performed on the cDNA prior to transfection and expression experiments. Deletion of the three basic pairs was performed according to standard procedures of oligonucleotide-directed mutagenesis. The pDX-based vector containing the modified cDNA was designated p594.

## EXAMPLE 3

## MODIFICATION OF THE PROTEIN C PROCESSING SITE

### A. Site Specific Mutagenesis

To enhance the processing of single-chain protein C to the two-chain form, two additional arginine residues were introduced into the protein, resulting in a cleavage site consisting of four basic amino acids. The resultant mutant precursor of protein C, designated PC962, contains the sequence Ser-His-Leu-Arg-Arg-Lys-Arg-Asp at the cleavage site between the light and heavy chains. Processing at the Arg-Asp bond results in a two-chain protein C molecule.

The mutant molecule was generated by altering the cloned cDNA by site-specific mutagenesis (essentially as described by Zoller and Smith, DNA 3:479-488, 1984) using the mutagenic oligonucleotide ZC962 (5' AGT CAC CTG AGA AGA AAA CGA GAC A 3') and oligonucleotide ZC550 (5' TCC CAG TCA CGA CGT 3'). Plasmid p594 was digested with Sst I, the approximately 840 bp fragment was cloned into M13mp11, and single-stranded template DNA was isolated. Following mutagenesis, a correct clone was identified by sequencing. Replicative form DNA was isolated and digested with Sst I, and the mutagenized fragment was recovered. This mutagenized fragment was joined with Sst I-cut p594 in a two-part ligation. Clones having the Sst I fragment inserted in the desired orientation were identified by restriction enzyme mapping. The resulting expression vector was designated pDX/PC962.

## EXAMPLE 4

## Expression and Characterization of Protein C

### A. Transfection of PDX/PC962 into 293 Cells

Ten μg of plasmid pDX/PC962 and 3 μg of plasmid pKO-neo (Southern and Berg, J. Mol. Appl. Genet. 1:327-341, 1982) were dissolved in a 525 μl aqueous solution containing 10 mM Tris-HCl pH 7.6 and 1 mM EDTA, followed by the addition of 75 μl of aqueous 2M $CaCl_2$. Next, 600 μl of 2X Hepes Buffered Saline (0.28 M NaCl, 50 mM Hepes, 1.5 mM sodium hydrogen phosphate, pH 7.2) was added dropwise with stirring, and the resulting solution was let stand for 20 minutes.

This solution was then poured onto 293 cells which were being cultivated in 10 cm diameter plates with Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal calf serum (FCS), 1 x PSN antibiotic mix (Gibco 600-

5640) and 2 mM L-glutamine. The cells were further cultured at 37°C and 5% $CO_2$ concentration for 6 hours to let salts containing the plasmids settle down onto the cells. The medium was then removed, and 2 ml of Hepes buffer solution containing 15% glycerol (pH 7.5) was added to the cells. The mixture was let stand for 2 minutes at room temperature to effect transfection.

The supernatant was removed from the cells, 10 ml D-MEM containing 10% FCS, 1 x PSN and 2 mM L-glutamine was added, and the cells were incubated at 37°C and 5% $CO_2$.

The transfected cells were grown for 48 hours in D-MEM containing 10% FCS, 1X PSN antibiotic mix (Gibco 600-5640), 2.0 mM L-glutamine and vitamin K (5 μg/ml).

The cells were removed from the plates by the addition of trypsin, then transferred to 15 cm diameter plates.

The cells were selected in 500 μg/ml G418 for 14 days, and the resulting colonies were screened by the immunofilter assay (McCracken and Brown, BioTechniques, 82-87, March/April 1984). The plates were rinsed with PBS or No Serum medium (D-MEM plus penicillin-streptomycin, 5 μg/ml vitamin K). Teflon™ mesh was then placed over the cells. Nitrocellulose filters were wetted with PBS or No Serum medium, as appropriate, and placed over the mesh. After four hours' incubation at 37°C, the filters were removed and placed in buffer A (50 mM Tris/HCl pH 7.4, 5mM EDTA, 0.05% NP-40, 150 mM NaCl, 0.25% gelatin) for 30 minutes at room temperature. The filters were incubated for one hour at room temperature, with shaking, in biotin-labeled sheep polyclonal antibody to protein C, 1 μg/ml in buffer A. The filters were then washed in buffer A and incubated for 1 hour at room temperature, with shaking, in avidin-conjugated horseradish peroxidase (Boehringer-Mannheim), 1:1000 in buffer A. Filters were washed in buffer B (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 M NaCl, 0.25% gelatin, 0.4% sarcosyl, 0.05% NP-40), then in $H_2O$, and incubated in color reagent (60 mg HRP color development reagent [Bio-Rad], 20 ml methanol, 100 μl $H_2O_2$ in 100 ml 50 mM Tris/HCl pH 7.4, 150 mM NaCl). The reaction was stopped by transferring the filters to $H_2O$. Six of the most intensely reacting colonies were picked by cylinder cloning and grown individually in 10-cm plates. When the cultures were nearly confluent, the protein C production levels were measured by ELISA. The results are given in Table 1 below.

TABLE 1

| Clone | pg/cell/day |
|---|---|
| 293/962 #1 | 1.8 |
| 293/962 #2 | 1.5 |
| 293/962 #3 | 2.0 |

B. Transfection of pDX/PC962 into BHK Cells

BHK 570 cells were cotransfected essentially as described above, but with the substitution of plasmid pSV2-DHFR (Subramani et al., *Mol. Cell Biol. 1*:845-864, 1981) for pKO-neo and methotrexate (250 nM) for G418 selection. Cells were screened for production of protein C, and protein C production was measured by ELISA. Expression levels for three clones are shown in Table 2.

TABLE 2

| Clone | pg/cell/day |
|---|---|
| BHK/2-1-5 | 0.39 |
| BHK/4-8 | 0.63 |
| BHK/4-10 | 0.61 |

EXAMPLE 5

Production of Activated Recombinant Protein C

Transfected 293 cells (293/962 #3) were cultured in serum-free ITES-eRDF medium (Table 3) for 3 days.

TABLE 3

ITES Concentration*

| | |
|---|---|
| Insulin | 9 μg/ml |
| Transferin | 10 μg/ml |
| Ethanolamine | 10 μM |
| Selenite | 20 nM |

* Concentration in eRDF (cf. Murakami et al; J. Agric. Chem. Soc. Japan 58:575, 1984).

Recombinant Protein C was purified from the serum-free culture supernatant of 293/962 #3 cells by use of an anti-Protein C monoclonal antibody (6H2) column. The 6H2 monoclonal antibody is characterized by its binding to protein C ("PC") in the presence of $Ca^{2+}$ ions and its failure to bind in the absence of $Ca^{2+}$ ions. The properties of this monoclonal antibody and the purification of PC by use of this antibody have been disclosed in published Japanese patent applications Nos. 61-134399 and 1-85091.

After filtering the supernatant liquid to remove cell debris, the supernatant was concentrated 20-40 fold with an ultrafilter membrane. The concentrate was then dialyzed against TBS (Tris buffered saline, 0.05 M Tris/HCl pH 7.4, 0.15 M NaCl). When not used immediately, the concentrate was stored at -40°C until immediately before use.

To about 250 ml of the above concentrate a 1 M $CaCl_2$ solution was added until the final concentration was about 5 mM $CaCl_2$. This concentrate was then placed on a Cellulofine™ (Biochemical Industry, Tokyo), column (1.5 cm x 5.0 cm) equilibrated with TBS containing 5 mM $CaCl_2$, and fixed with anti-Protein C monoclonal antibody 6H2 which adsorbed the recombinant Protein C.

The column was then thoroughly washed with 0.05 M Tris/HCl pH 7.4, 1.0 M NaCl, 5 mM $CaCl_2$ buffer. After confirmation that the absorbance of the effluent at a wavelength of 280 nm was 0.01 or less, the rPC bound to the column was eluted with a 0.05 M Tris/HCl pH 7.4, 0.15 M NaCl, 10 mM $Na_2EDTA$ buffer.

The rPC eluted from the column as a single peak. Electrophoresis on a 20% SDS-polyacrylamide gel under nonreducing conditions indicated that the protein was in a single band. This process produced about 10.7 mg of rPC from about 15 mg of starting material (approximately 71% yield).

B. Activation of Recombinant Protein C

(1) Activating Reaction

For activation, the rPC solution was concentrated to 2 mg/ml using an Amicon concentrator with a YM10 ultramembrane filter (Amicon, Danvers, Mass.) and dialyzed against a 0.05 M Tris/HCl pH 7.5, 0.15 M NaCl buffer solution. To 1860 µl of the above 2 mg/ml rPC solution were added 69 µl of aqueous 2.7 mg/ml bovine thrombin solution (Mochida Seiyaku, Tokyo), 6 µl of aqueous 500 mM EDTA, and 1065 µl of TBS to give a total volume of 3 ml. After

thorough mixing, the mixture was incubated in a water bath at 37°C for 3 hours to activate the recombinant protein C.

(2) Purification of Activated Recombinant Protein C (ArPC)

Thrombin was removed and ArPC was purified from the activation mixture by use of a cation exchange column and an anion exchange column.

(a) Cation Exchange Column

The ArPC solution was diluted twofold by the addition of 3 ml of a 0.02 M MES (2-(N-morpholino) ethane sulfuric acid)/Tris pH 6.0, 0.05 M NaCl buffer solution. The diluted solution was applied to a 1.5 cm x 4 cm S-Sepharose Fast Flow column (Pharmacia) equilibrated with the same buffer. After washing the column with the same buffer solution, the fraction adsorbed to the column was eluted with a linear gradient of 0.05 to 1.0 M NaCl, to give a pass-through fraction, washing fraction, and concentration fractions. The ArPC was contained in the pass-through fraction and the 0.3-0.5 M NaCl fractions. The thrombin was eluted at about 0.6 M NaCl. The ArPC and the thrombin were separated almost completely by this operation. The fractions containing ArPC were pooled together, and about 30 ml of this solution was added to an equal quantity of 0.02 M MES/Tris pH 6.0 buffer to reduce the concentration of NaCl.

(b) Anion Exchange Column

The ArPC solution (60 ml) obtained in the preceding step was applied to a 1.0 cm x 3.6 cm Q-Sepharose Fast Flow column (Pharmacia) equilibrated with a 0.02 M MES/Tris pH 6.0, 0.05 M NaCl buffer solution. After equilibrating the column with the above buffer solution, the ArPC adsorbed to the column was eluted with 0.02 M MES/Tris pH 6.0 buffer containing 0.4 M NaCl. This gave a single peak containing ArPC (by absorbance analysis at 280 nm). The ArPC was present as a single band on a 10%-20% gradient SDS-PAGE under nonreducing conditions.

## EXAMPLE 6

## Activation of Plasma Protein C

(A) Activation of Human Plasma Protein C (pPC)

Purified human protein C (obtained from Enzyme Research Laboratories, Inc., South Bend, Ind.) [(Lot HPC 220)] was concentrated by use of

an ultrafilter membrane (Amicon YM 10), and dialyzed against 0.05 M Tris/HCl pH 7.4, 0.15 M NaCl buffer (TBS). To 865 μl of the resultant 2.2 mg/ml pPC solution were added 34 μl of 2.7 mg/ml aqueous bovine thrombin solution (Mochida Seiyaku, Tokyo), 3 μl of aqueous 500 mM EDTA, and 598 μl of TBS to give a total volume of 1.5 ml. After thorough mixing, the mixture was incubated for 3 hours in a water bath at 37°C to give activated plasma protein C (ApPC).

(B) Purification of Activated Human Plasma Protein C

Activated plasma protein C (ApPC) was purified from the mixed solution containing ApPC after the activating reaction using the method described above for ArPC purification.

A single peak (absorbance analysis at 280 nm) containing ApPC was obtained and shown to be a single band by 10-20% gradient SDS-PAGE under nonreducing conditions.

## EXAMPLE 7

## Amino Acid Analysis of the Carboxy-Terminus of the Activated Protein C (APC) Light Chain and Proportional Measurement

The heavy- and light-chains of purified APC were separated from each other by reduction of the connecting S-S bond. About 560 μg (measured based on the absorbance of PC) of APC was incubated for 6 hours in 3 ml of 6 M guanidine hydrochloride-0.5M Tris buffer solution (pH 8.3) containing 10 mg of dithiothreitol in a $N_2$ gas atmosphere at 65°C. Two hundred and fifty μl of 43 mg/ml monoiodoacetic acid aqueous solution (pH 4-6) was added, and this reaction solution is incubated at 37°C for 30 minutes to convert the -SH groups to $-SCH_2COOH$ groups to prevent disulfidation. The resultant low-molecular weight solute was permeated through an ultrafilter membrane (Amicon Co.) while gradually adding about 10 volumes of water to the solution side to keep the volume of the solution nearly constant.

The aqueous solution of mixed heavy- and light-chain proteins was fractionated by reversed-phase partition chromatography using a 6.2 mm x 80 mm Poly-F column (Du Pont Co.) under the conditions shown in Table 4.

TABLE 4

| | | | |
|---|---|---|---|
| Solvent A: | 0.1 M $NH_4HCO_3$ (pH 8) | | |
| Solvent B: | Acetonitrile | | |
| Flow rate: | 0.5 ml/min | | |
| Gradient: | Time (min) | B (vol%) | |
| | 0 | 0 | |
| | 5 | 0 | ] Linear gradient (5 vol%/min) |
| | 25 | 100 | |
| | 30 | 100 | |
| Detection: | 215 nm | | |
| Column temperature: | room temperature | | |

The fraction containing the isolated light-chain (as determined by the presence of an $M_r \simeq 20{,}000$ band on a 10%-20% gradient SDS-polyacrylamide gel) was freeze dried at -80°C and dissolved in 200 µl of 0.1 M urea, 50 mM phosphoric acid buffer solution (pH 8.0). 0.6 µg (about 4 wt% aqueous solution) of Endoproteinase Asp-N (Boehringer Mannheim) was added, and the mixture was incubated at 37°C for 6 hours to cut the peptide bond on the amino-terminal side of the aspartic acid in the light chain.

The resultant mixture of light-chain peptide fragments was fractionated by reversed-phase partition chromatography using an octadecyl silanol (ODS) column under the conditions shown in Table 5.

TABLE 5

| | |
|---|---|
| Column: | Vydac C18 column (The Separations Group, Hesperia, CA) (4.6 mm x 250 mm long, SUS) [218 TP54] |
| Solvent A: | 0.1 vol% trifluoroacetic acid, 1 vol% acetonitrile in $H_2O$ |
| Solvent B: | 0.1 vol% trifluoroacetic acid + 99.9 vol% acetonitrile |
| Flow rate: | 0.5 ml/min |

Column temperature: Room temperature

| Gradient: | Time (min) | B (vol%) | |
|---|---|---|---|
| | 0 | 0 | |
| | 5 | 0 | ] Linear gradient |
| | 95 | 45 | (0.5 vol%/min) |
| | 100 | 100 | |

Detection: 215 nm

The peptide peaks were sequenced by use of a Model 477A protein sequencer (Applied BioSystems, Inc., Foster City, Calif.). Peptides having an amino terminus at amino acid 128 were generally present among the peaks having a retention time of 60 to 70 minutes on the C18 column.

Amino acid sequence analysis indicated the presence of two different light chain carboxy-terminal amino acids, lysine (amino acid No. 150) and arginine (amino acid No. 152). Since the enzyme Endo-proteinase Asp-N has no activity to cut the bonds between amino acid Nos. 150, 151, 152 and 153, it was concluded that the carboxy-termini of the light chains of activated protein C produced as described above are lysine and arginine.

Based on the results of amino acid sequence analysis, the amount (pmol) of light-chain peptide 128-150 was determined by subtracting the amounts of other peptides contained in the same peak. The amount of peptide was determined from the molar ratio of each amino acid in the earliest cycle in which amino acids showing the same degree of recovery were detected. The amount of light-chain peptide 128-152 was determined in the same manner. The molar ratio of peptide 128-150 to peptide 128-152 ($APC^{150}/APC^{152}$) was found to be 1:3.

## EXAMPLE 8

### Activation of Plasma Protein C

(A) Activation of Human Plasma Protein C (pPC)

Human protein C purified by using a monoclonal antibody column was concentrated by use of an ultrafilter membrane (Amicon YM 10), and dialyzed against 0.05 M Tris/HCL pH 7.4, 0.15 M NaCl buffer (TBS). To 450 µl

of the resultant 2 mg/ml pPC solution were added 17 μl of 2.7 mg/ml aqueous bovine thrombin solution (Mochida Seiyaku, Tokyo), 2 μl of aqueous 500 mM EDTA, and 531 μl of TBS to give a total volume of 1.0 ml. After thorough mixing, the mixture was incubated for 5 hours in a water bath at 37°C to give activated plasma protein C(ApPC).

(B) Purification of Activated Human Plasma Protein C

Activated plasma protein C(ApPC) was purified from the mixed solution containing ApPC after the activating reaction using the method described above for ArPC purification.

A single peak (absorbance analysis at 280 nm) containing ApPC was obtained and shown to be a single band by 10-20% gradient SDS-PAGE under nonreducing conditions.

## EXAMPLE 9

## Amino Acid Analysis of the Carboxy-Terminus of the Activated Plasma Protein C(ApPC) Light Chain and Proportional Measurement

The heavy- and light-chain of ApPC were separated from each other by reduction on the connecting S-S bond. About 550 μg (measured based on the absorbance of PC) of APC was incubated for 4 hours in 3 ml of 6 M guanidine hydrochloride-0.5M Tris buffer solution (pH 8.3) containing 10 mg of dithiothreitol in a $N_2$ gas atmosphere at 65°C. Two hundred and fifty microliters of 43 mg/ml monoiodoacetic acid aqueous solution (pH 4-6) was added, and this reaction solution was incubated at 37°C for 30 minutes to convert the -SH groups to -$SCH_2COOH$ groups to prevent disulfidation. The resultant low-molecular weight solute was permeated through an ultrafilter membrane (Amicon Co.) while gradually adding about 10 volumes of water to the solution side to keep the volume of the solution nearly constant.

The aqueous solution of mixed heavy- and light-chain proteins was fractionated by reversed-phase partition chromatography using a 6.2 mm x 80 mm Poly-F column (DuPont Co.) under the conditions shown in Table 6.

TABLE 6

Solvent A: 0.1 M $NH_4HCO_3$(pH8)

Solvent B: Acetonitrile

Flow rate: 0.5 ml/min

Column temperature: Room temperature

| Gradient: | Time (min) | B (vol%) | |
|---|---|---|---|
| | 0 | 0 | |
| | 5 | 0 | ] Linear gradient |
| | 25 | 100 | (5 vol%/min) |
| | 30 | 100 | |

Detection: 215 nm

Column temperature: Room temperature

The fraction containing the isolated light-chain (as determined by the presence of an $M_t$≈20,000 band on a 10%-20% gradient SDS-polyacrylamide gel) was freeze dried at -80°C and dissolved in 200 µl of 0.1 M urea, 50 mM phosphoric acid buffer solution (pH 8.0). 0.6 µg (about 4 wt% aqueous solution) of Endoproteinase Asp-N (Boehringer Mannheim) was added, and the mixture was incubated at 37°C for 6 hours to cut the peptide bond on the amino-terminal side of the aspartic acid in the light chain.

The resultant mixture of light-chain peptide fragments was fractionated by reversed-phase partition chromatography using an octadecyl silanol (ODS) column under the conditions shown in Table 7.

TABLE 7

Column: Vydac C18 column (The Separations Group, Hesperia, CA) (4.6 mm x 250 mm long, SUS) [218 TP54]

Solvent A: 0.1 vol% trifluoroacetic acid, 1 vol% acetonitrile in $H_2O$

Solvent B: 0.1 vol% trifluoroacetic acid + 99.9 vol% acetonitrile

Flow rate: 0.5 ml/min

Column temperature: Room temperature

Gradient:

| Time (min) | B (vol%) | |
|---|---|---|
| 0 | 0 | |
| 5 | 0 | ] Linear gradient (0.5 vol%/min) |
| 95 | 45 | |
| 100 | 100 | |

Detection: 215 nm

Peptides having an amino terminus at amino acid 128 were generally present among the peaks having a retention time of 60 to 70 minutes on the C18 column.

Chymotrypsin Proteolysis

The fraction of the peaks having the retention time of 60 to 70 minutes on the C18 column was taken out, freeze dried at -80°C and dissolved in 100 µl of 100 mM phosphate buffer (pH 7.0).

In order to cut the peptide bond on the carboxy-terminal side of the tryptophan, tryosine, etc., in the above fraction, 5.5 ng [500 µg/ml phosphate buffer (pH 7.0)] of chymotrypsin (Sigma, C-3142) was added thereto, and the mixture was incubated at 37°C for 4 hours.

The resultant mixture of peptide fragments was fractionated by reversed-phase partition chromatography using an octadecyl silanol (ODS) column under the same conditions as shown in Table 7.

Analysis of amino acid sequence of the peptide fragment obtained above was performed by use of a Model 477A protein sequencer, and amino acid

analysis was performed by PTC amino acid analysis method (R. L. Heinrikson, S. C. Meredich, Anal. Biochem. 136:65, 1984; D. Antherton, "Techniques in Protein Chemistry," ed. by T. E. Hugli, P. 273, Academic Press, New York, 1989).

A peptide having lysine at amino acid number 146 on the amino terminus was anticipated to be eluted in 24-40 minutes with the C18 column.

The results of amino acid sequence analysis and amino acid analysis indicated the presence of three different light chain carboxy-terminal amino acids, lysine (amino acid number 150), lysine (amino acid number 151) and arginine (amino acid number 152).

Since chymotrypsin has no activity to cut the bonds between amino acid numbers 149, 150, 151, 152 and 153, it was concluded that the carboxy-termini of the light chains of activated protein C produced as described above are lysine (amino acid number 150), lysine (amino acid number 151) and arginine (amino acid number 152) as mentioned above.

The amount (pico moles) of the 146-150 peptide of the light chain was determined from the recovery of the first residue lysine in the amino acid sequence analysis or from the content of each amino acid in the amino acid analysis. The amount of the 146-151 peptide and the 146-152 peptide of light chain were determined in the same manner. The molar ratio of peptide 146-150, 146-151 to 146-152 ($APC^{150}/APC^{151}/APC^{152}$) was found to be 1:2:2.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

## Claims

1. Activated human protein C having a heavy chain and a light chain, said light chain consisting essentially of an amino acid sequence selected from the group consisting of the amino acid sequence of Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 150; the amino acid sequence of Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 151; and the amino acid sequence of Figure 1 from alanine, amino acid number 1, to arginine, amino acid number 152.

2. Activated human protein C according to claim 1 produced by activating recombinant protein C.

3. Activated human protein C according to claim 1 produced by activating plasma protein C.

4. Activated human protein C according to claim 1 wherein said protein C is recombinant activated protein C.

5. A composition comprising a first form of activated human protein C, a second form of activated human protein C and/or a third form of activated human protein C, said first form comprising a heavy chain disulfide bonded to a light chain having the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 150; said second form comprising a heavy chain disulfide bonded to a light chain having the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to lysine, amino acid number 151; and said third form comprising a heavy chain disulfide bonded to a light chain having the amino acid sequence shown in Figure 1 from alanine, amino acid number 1, to arginine, amino acid number 152.

6. A composition according to claim 5 wherein the ratio of said first form to said second or third form is between about 1:10 and 10:1, inclusive.

7. A composition according to claim 5 wherein the ratio of said first form to said second or third form is between 1:5 and 5:1, inclusive.

8. A composition according to claim 5 wherein the ratio of said first form to said third form is about 1:3.

9. A composition according to claim 5 wherein the ratio of said first form to said second form is about 3:1.

10. A composition according to any of claims 5 through 9 wherein said ratio is determined by a method comprising:

(a) separating the light chains from the heavy chains;
(b) fragmenting the separated light chains to produce polypeptides;
(c) fractionating the polypeptides;
(d) sequencing the fractionated polypeptides; and
(e) calculating the molar ratio of said first form to said second form.

11. A composition according to claim 10 wherein said separating step comprises reduction of disulfide bonds between said heavy chains and said light chains to produce -SH groups and modification of said -SH groups to prevent subsequent disulfidation.

12. A composition according to claim 10 wherein said fragmenting step comprises proteolysis with endoproteinase Asp-N, optionally followed by proteolysis with chymotrypsin after separation of a peptide.

13. A composition according to claim 10 wherein said fractionating step comprises high performance liquid chromatography.

14. A composition according to claim 5 wherein said first, second and third forms are derived from recombinant human protein C.

15. A composition according to claim 5 wherein said first, second and/or third forms are forms of recombinant activated human protein C.

16. A composition according to claim 5 wherein said first, second and/or third forms are derived from activated plasma protein C.

17. A method for producing a composition comprising human activated protein $C^{150}$, human activated protein $C^{151}$ and human activated protein $C^{152}$ from human protein C comprising the steps of:

(a) concentrating the human protein C;

(b) reducing the salt concentration of the concentrated protein C to produce a reduced salt solution;

(c) exposing the reduced salt solution to thrombin to activate the protein C;

(d) purifying the activated protein C from the thrombin by subjecting the product of step (c) to ion exchange chromatography to produce one or more fractions containing purified activated protein C; and

(e) collecting the fraction(s) containing the purified activated protein C, wherein said purified activated protein C consists essentially of activated protein $C^{150}$, activated protein $C^{151}$ and activated protein $C^{152}$.

18. A method according to claim 17 wherein the step of exposing comprises combining said reduced salt solution with thrombin to produce a ratio of protein C to thrombin of from 1:1 to 200:1 by weight.

19. A method according to claim 18 wherein said ratio is approximately 20:1.

20. A method according to claim 17 wherein said human protein C is concentrated to between about 2.0 to 2.5 mg/ml.

21. A method according to claim 17 wherein the step of purifying comprises cation exchange chromatography followed by anion exchange chromatography.

22. A method according to claim 17 wherein said thrombin is bovine $\gamma$-thrombin containing less than 10% bovine $\beta$- or $\gamma$-thrombin.

23. A method according to claim 17 , wherein said human protein C is plasma protein C.

24. A method according to claim 17 wherein said human protein C is recombinant protein C.

25. A method according to claim 24 wherein said recombinant protein C is produced by culturing mammalian cells transfected to express a protein C precursor having the sequence $R_1$-$R_2$-$R_3$-$R_4$ at the junction between the light chain

and the activation peptide, wherein each of $R_1$ through $R_4$ is a lysine residue or an arginine residue.

26. A method according to claim 25 wherein said cells are BHK cells or 293 cells.

27. A method according to claim 25 wherein said cells are cultured in a medium containing vitamin K.

28. A method according to claim 25 wherein said cells are cultured in a medium that is essentially free of serum.

29. A method according to claim 17 wherein said composition comprises a ratio of activated protein $C^{150}$ to activated protein $C^{152}$ between about 1:10 and 10:1, inclusive.

30. A method according to claim 17 wherein said composition comprises a ratio of activated protein $C^{150}$ to activated protein $C^{151}$ or activated protein $C^{152}$ between about 1:5 and 5:1, inclusive.

31. A method according to claim 17 wherein said composition comprises a ratio of activated protein $C^{150}$ to activated protein $C^{152}$ of about 1:3 and/or a ratio of activated protein $C^{150}$ to activated protein $C^{151}$ of about 3:1.

32. A pharmaceutical composition containing a physiologically acceptable carrier or diluent and activated plasma protein C, wherein said activated human protein C has a heavy chain and a light chain, said light chain consisting essentially of the amino acid sequence of Figure 1, from alanine, amino acid number 1, to lysine, amino acid number 150.

33. A pharmaceutical composition containing a physiologically acceptable carrier or diluent and activated plasma and/or recombinant protein C, wherein said activated plasma and/or recombinant protein C has a heavy chain and a light chain, said light chain consisting essentially of the amino acid sequence of Figure 1, from alanine, amino acid number 1, to lysine, amino acid number 150.

34. A pharmaceutical composition containing a physiologically acceptable carrier or diluent and activated human protein C, wherein said activated human protein C has a heavy chain and a light chain, said light chain consisting essentially of the amino acid sequence of Figure 1, from alanine, amino acid number 1, to lysine, amino acid number 151.

35. A pharmaceutical composition containing a physiologically acceptable carrier or diluent and activated plasma protein C, wherein said activated human protein C has a heavy chain and a light chain, said light chain consisting essentially of the amino acid sequence of Figure 1, from alanine, amino acid number 1, to arginine, amino acid number 152.

36. A pharmaceutical composition containing a physiologically acceptable carrier or diluent and activated plasma and/or recombinant protein C, wherein said activated plasma and/or recombinant protein C has a heavy chain and a light chain, said light chain consisting essentially of the amino acid sequence of Figure 1, from alanine, amino acid number 1, to arginine, amino acid number 152.

37. A pharmaceutical composition containing a physiologically acceptable carrier or diluent and activated protein $C^{150}$, activated protein $C^{151}$ and/or activated protein $C^{152}$ in a ratio of about 1:10 to 10:1.

38. The pharmaceutical composition of claim 37 wherein the ratio is in the range of about 1:5 to 5:1.

39. The pharmaceutical composition of claim 37 wherein the ratio of activated protein $C^{150}$ to activated protein $C^{151}$ is about 3:1 and/or the ratio of activated protein $C^{150}$ to activated protein $C^{152}$ is about 1:3.

40. A pharmaceutical composition containing a physiologically acceptable carrier or diluent and activated protein $C^{151}$ and activated protein $C^{152}$.

41. A method for determining the ratio of activated protein $C^{150}$ to activated protein $C^{151}$ and activated protein $C^{152}$ in purified activated protein C, comprising:

(a) reducing a disulfide bond which connects the heavy chain and the light chain of activated protein C to an -SH group;

(b) modifying the -SH group such that further disulfidation is prevented;

(c) separating the light chain from the heavy chain;

(d) fragmenting the light chain;

(e) fractionating the fragmented light chain;

(f) sequencing the fractionated light chain; and

(g) calculating the molar ratio of activated protein $C^{150}$ to activated protein $C^{151}$ and activated protein $C^{152}$.

42. The method of claim 41 wherein the step of reducing comprises treating the activated protein C with dithiothreitol in 6 M guanidine-HCl, pH 8.

43. The method of claim 41 wherein the step of modifying comprises treating the -SH group with monoiodoacetic acid at pH 4 - pH 6.

44. The method of claim 41 wherein the step of separating comprises reversed-phase partition chromatography.

45. The method of claim 41 wherein the step of fragmenting comprises proteolysis with endoproteinase Asp-N.

46. The method of claim 44 wherein the step of fragmenting further comprises proteolysis with chymotrypsin after separation of a peptide.

47. The method of claim 41 wherein the step of fractionating comprises high performance liquid chromatography.

FIG. 1A

```
GGCTGTCATG GCGGCAGGAC GGCGAACTTG CAGTATCTCC ACGACCCGCC CCTGTGCCAG TGCCTCCA

-42      -40                                                  -30
ATG TGG CAG CTC ACA AGC CTC CTG CTG TTC GTG GCC ACC TGG GGA ATT TCC GGC
MET Trp Gln Leu Thr Ser Leu Leu Leu Phe Val Ala Thr Trp Gly Ile Ser Gly

                 -20                                                -10
ACA CCA GCT CCT CTT GAC TCA GTG TTC TCC AGC AGC GAG CGT GCC CAC CAG GTG
Thr Pro Ala Pro Leu Asp Ser Val Phe Ser Ser Ser Glu Arg Ala His Gln Val

                        -1  +1                                        10
CTG CGG ATC CGC AAA CGT GCC AAC TCC TTC CTG GAG GAG CTC CGT CAC AGC AGC
Leu Arg Ile Arg Lys Arg Ala Asn Ser Phe Leu Glu Glu Leu Arg His Ser Ser

                                20                                      30
CTG GAG CGG GAG TGC ATA GAG GAG ATC TGT GAC TTC GAG GAG GCC AAG GAA ATT
Leu Glu Arg Glu Cys Ile Glu Glu Ile Cys Asp Phe Glu Glu Ala Lys Glu Ile

                                    40
TTC CAA AAT GTG GAT GAC ACA CTG GCC TTC TGG TCC AAG CAC GTC GAC GGT GAC
Phe Gln Asn Val Asp Asp Thr Leu Ala Phe Trp Ser Lys His Val Asp Gly Asp

     50                                                 60
CAG TGC TTG GTC TTG CCC TTG GAG CAC CCG TGC GCC AGC CTG TGC TGC GGG CAC
Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys Gly His
```

```
                                    80
GGC ACG TGC ATC GAC GGC ATC GGC AGC TTC AGC TGC GAC TGC CGC AGC GGC TGG
Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys Arg Ser Gly Trp

                                    90                              100
GAG GGC CGC TTC TGC CAG CGC GAG GTG AGC TTC CTC AAT TGC TCG CTG GAC AAC
Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu Asn Cys Ser Leu Asp Asn

                                   110                             120
GGC GGC TGC ACG CAT TAC TGC CTA GAG GAG GTG GGC TGG CGG CGC TGT AGC TGT
Gly Gly Cys Thr His Tyr Cys Leu Glu Glu Val Gly Trp Arg Arg Cys Ser Cys

                                   130
GCG CCT GGC TAC AAG CTG GGG GAC GAC CTC CTG CAG TGT CAC CCC GCA GTG AAG
Ala Pro Gly Tyr Lys Leu Gly Asp Asp Leu Leu Gln Cys His Pro Ala Val Lys

                                   140                             150
TTC CCT TGT GGG AGG CCC TGG AAG CGG ATG GAG AAG AAG CGC AGT CAC CTG AAA
Phe Pro Cys Gly Arg Pro Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys

                                   160                             170
CGA GAC ACA GAA GAC CAA GAA GAC CAA GTA GAT CCG CGG CTC ATT GAT GGG AAG
Arg Asp Thr Glu Asp Gln Glu Asp Gln Val Asp Pro Arg Leu Ile Asp Gly Lys
```

FIG. 11B

FIG. 1C

```
                          180                                       190
ATG ACC AGG CGG GGA GAC AGC CCC TGG CAG GTG GTC CTG CTG GAC TCA AAG AAG
Met Thr Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys

                                   200                                  210
AAG CTG GCC TGC GGG GCA GTG CTC ATC CAC CCC TCC TGG GTG CTG ACA GCG GCC
Lys Leu Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr Ala Ala

                                        220
CAC TGC ATG GAT GAG TCC AAG AAG CTC CTT GTC AGG CTT GGA GAG TAT GAC CTG
His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly Glu Tyr Asp Leu

    230                                                 240
CGG CGC TGG GAG AAG TGG GAG CTG GAC CTG GAC ATC AAG GAG GTC TTC GTC CAC
Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile Lys Glu Val Phe Val His

      ♦       250                                           260
CCC AAC TAC AGC AAG AGC ACC ACC GAC AAT GAC ATC GCA CTG CTG CAC CTG GCC
Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn Asp Ile Ala Leu Leu His Leu Ala

                          270                                       280
CAG CCC GCC ACC CTC TCG CAG ACC ATA GTG CCC ATC TGC CTC CCG GAC AGC GGC
Gln Pro Ala Thr Leu Ser Gln Thr Ile Val Pro Ile Cys Leu Pro Asp Ser Gly
```

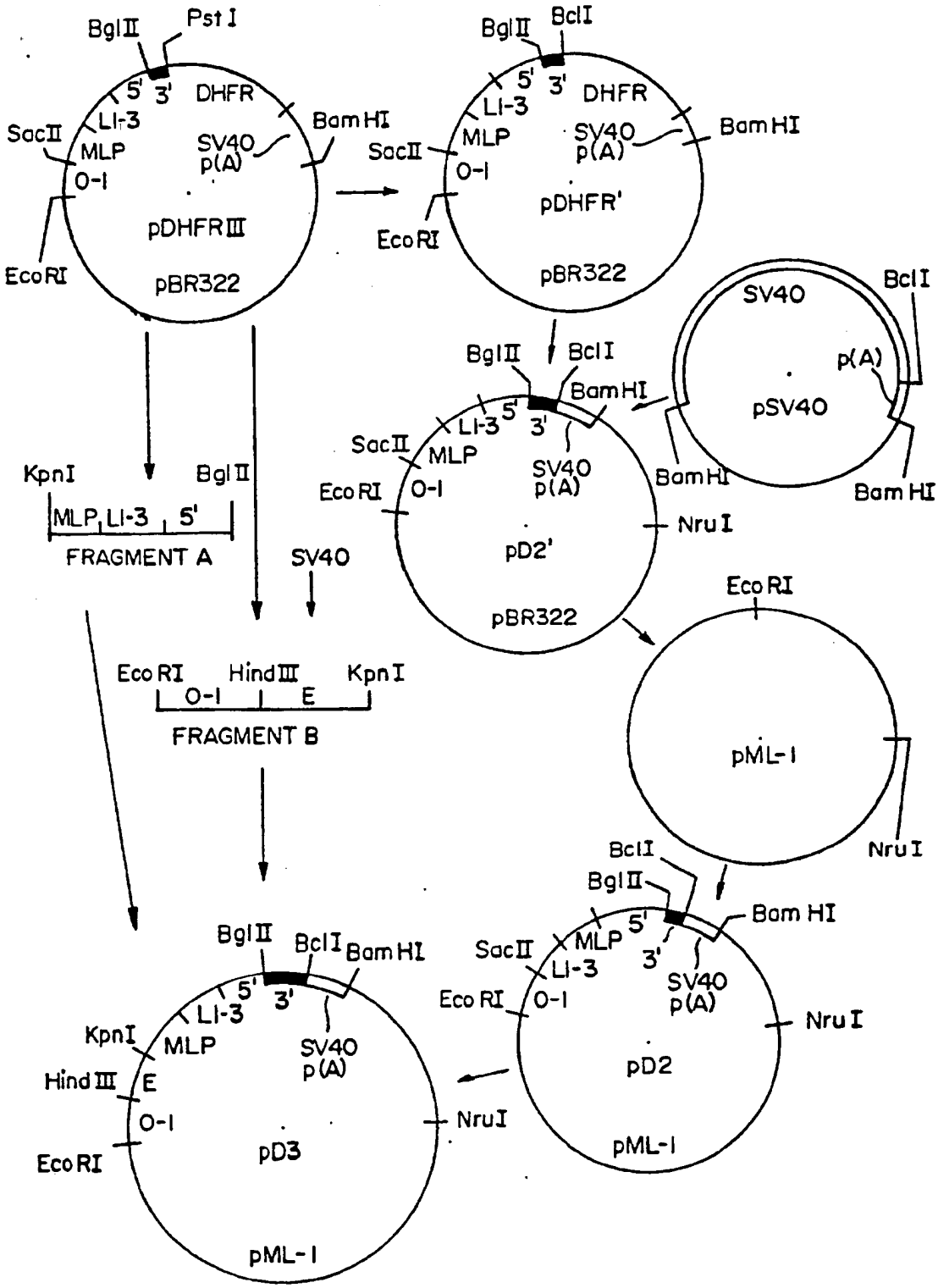
```
                                    290                                 300
CTT GCA GAG CGC GAG CTC AAT CAG GCC GGC CAG GAG ACC CTC GTG ACG GGC TGG
Leu Ala Glu Arg Glu Leu Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp

                                        310
GGC TAC CAC AGC AGC CGA GAG AAG GAG GCC AAG AGA AAC CGC ACC TTC GTC CTC
Gly Tyr His Ser Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu

    320                                         330
AAC TTC ATC AAG ATT CCC GTG GTC CCG CAC AAT GAG TGC AGC GAG GTC ATG AGC
Asn Phe Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser

            340                                         350
AAC ATG GTG TCT GAG AAC ATG CTG TGT GCG GGC ATC CTC GGG GAC CGG CAG GAT
Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg Gln Asp

                        360                                 370
GCC TGC GAG GGC GAC AGT GGG GGG CCC ATG GTC GCC TCC TTC CAC GGC ACC TGG
Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe His Gly Thr Trp

                                    380                                 390
TTC CTG GTG GGC CTG GTG AGC TGG GGT GAG GGC TGT GGG CTC CTT CAC AAC TAC
Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys Gly Leu Leu His Asn Tyr

                                        400
GGC GTT TAC ACC AAA GTC AGC CGC TAC CTC GAC TGG ATC CAT GGG CAC ATC AGA
Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu Asp Trp Ile His Gly His Ile Arg
```

FIG. 1D

```
    410                                                 419
GAC AAG GAA GCC CCC CAG AAG AGC TGG GCA CCT TAG CGACCCTCCC TGCAGGGCTG
Asp Lys Glu Ala Pro Gln Lys Ser Trp Ala Pro

GGCTTTTGCA TGGCAATGGA TGGGACATTA AAGGGACATG TAACAAGCAC ACCGGCCTGC TGTTCTGTCC

TTCCATCCCT CTTTTGGGCT CTTCTGGAGG GAAGTAACAT TTACTGAGCA CCTGTTGTAT GTCACATGCC

TTATGAATAG AATCTTAACT CCTAGAGCAA CTCTGTGGGG TGGGGAGGAG CAGATCCAAG TTTTGCGGGG

TCTAAAGCTG TGTGTGTTGA GGGGGATACT CTGTTTATGA AAAAGAATAA AAAACACAAC CACGAAAAAA
```

FIG. 1E

FIG. 2



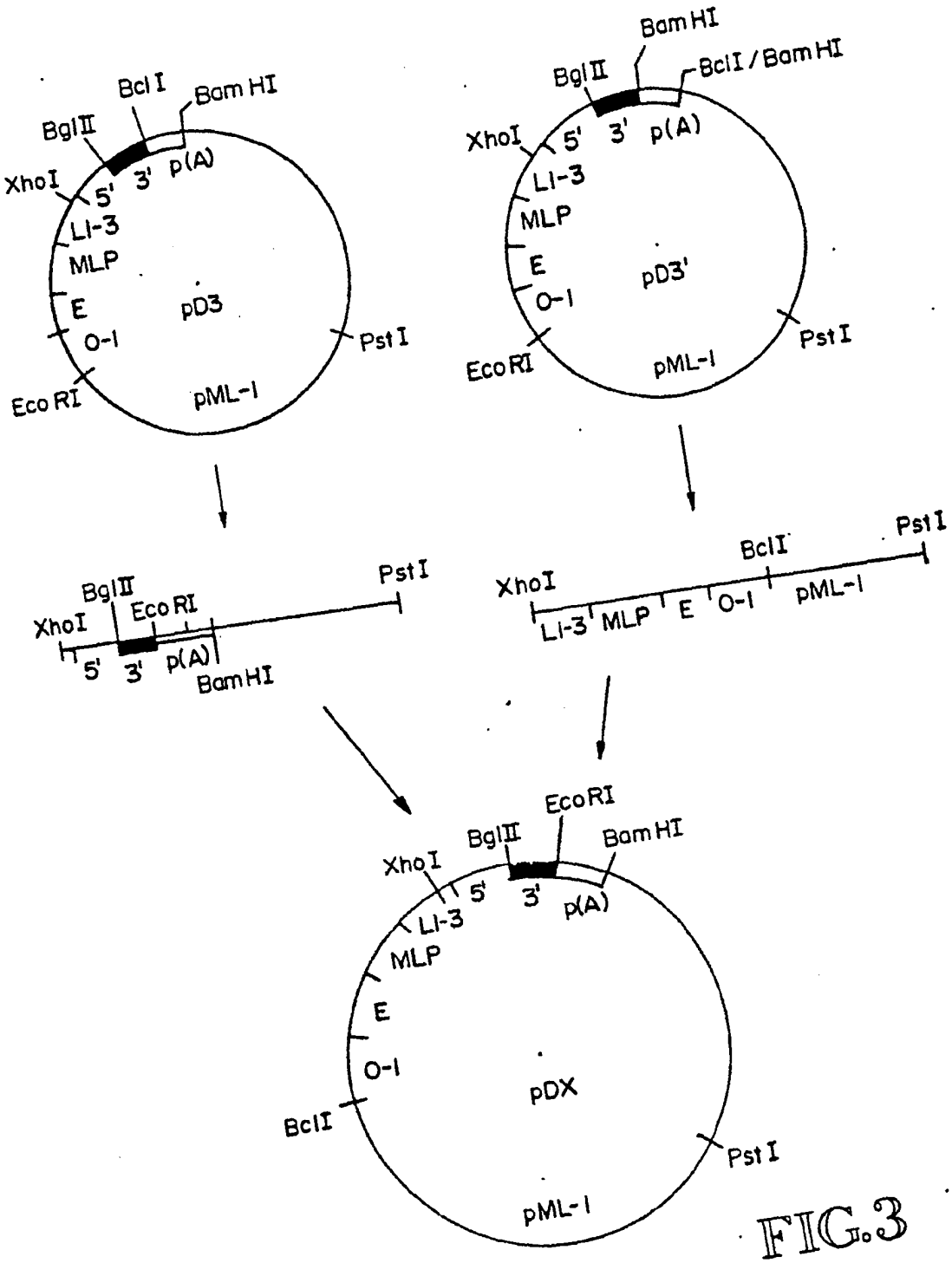

BclI
BamHI
BglII
XhoI
5'
3'
p(A)
LI-3
MLP
E
O-1
pD3
PstI
EcoRI
pML-1
BamHI
BglII
BclI / BamHI
pD3'
BglII
EcoRI
BamHI
pDX

FIG. 3